

## Characterization of reference gene expression in tung tree (*Vernicia fordii*)



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### ABSTRACT

Tung oil from tung tree (*Vernicia fordii*) is widely used as a drying ingredient in paints, varnishes, and other coatings and finishes. Recent research has focused on the understanding of the biosynthesis of oil in tung trees. Many oil biosynthetic genes have been identified in tung tree but little is known about the expression patterns of the genes in tung seeds. Quantitative real-time-PCR (qPCR) assays are widely used for gene expression analysis. One crucial task of qPCR assay design is to select stably expressed internal reference genes for data analysis. The objective of this study was to characterize the expression of potential reference genes in the tung tree to provide a sound basis for reliable and reproducible qPCR results. The expression of tung 60s ribosome protein L19 (Rpl19b), glyceraldehyde 3 phosphate dehydrogenase (Gapdh) and ubiquitin protein ligase (Ubl) was examined by TaqMan and SYBR Green qPCR using RNA from three trees, three tissues (seeds, leaves and flowers) and 11 time events of developing seeds. The variations of the three mRNA levels were compared between two RNA extraction methods, two cDNA preparations, and the same or different PCR plates. Overall results demonstrated that Rpl19b was the most stably expressed gene, followed closely by Ubl, and Gapdh was the worst among the three genes under optimized qPCR assay conditions. These results suggest that Rpl19b and Ubl are preferable reference genes for both TaqMan and SYBR Green qPCR assays. The development of these reference genes for quantitative gene expression analyses in tung trees should facilitate identifying target genes for genetic engineering industrial oils in oilseed crops.

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### 1. Introduction

The tung tree (*Vernicia fordii*) is a tropical plant with a very limited growing area in the United States (Abbott, 1929; Potter, 1968). Most of the tung orchards in the southern United States were destroyed by several hurricanes including Hurricanes Betsy in 1965, Camille in 1969, and Katrina and Rita in 2005 (<http://www.waterlox.com/uploads/docs/Tung-oil-hotlink-story-REVISED-2.pdf>). Tung tree produces industrially important tung oil in the seeds which contain approximately 50–60% oil (dry weight basis) with approximately 80 mole percent  $\alpha$ -eleostearic acid (9cis, 11trans, 13trans octadecatrienoic acid) (Sonntag, 1979). Tung oil is widely used as a drying ingredient in paints, varnishes, coatings and finishes due to its ability to be readily oxidized owing to the three unique conjugated double bonds in eleostearic acid (Kopacz,

1968; Pryde, 1979). Recently, tung oil have been used as a raw material to produce biodiesel (Park et al., 2008; Chen et al., 2010c, 2012b; Manh et al., 2012; Shang et al., 2012), polyurethane and wood flour composites (Aranguren et al., 2012), thermosetting polymer (Liu et al., 2013), and repairing agent for self-healing epoxy coatings (Samadzadeh et al., 2011).

In the past ten years, research has focused on creating transgenic plants that can produce tung oil and much of the emphasis has been on the understanding of the biosynthesis of oil in tung trees. Many oil biosynthetic genes have been identified in tung tree, including FADX, the diverged FAD2-like enzyme that catalyzes the formation of  $\alpha$ -eleostearic acid from linoleic acid (Dyer et al., 2002), diacylglycerol acyltransferases (DGAT), a group of hydrophobic membrane proteins responsible for the last and rate-limiting step of triacylglycerol (TAG) biosynthesis in eukaryotic organisms (Shockley et al., 2006; Cao, 2011; Liu et al., 2012; Chen and Smith, 2012), acyl-CoA binding proteins (Pastor et al., 2013), cytochrome b5 (Hwang et al., 2004), cytochrome b5 reductase (Shockley et al., 2005), glycerol-3-phosphate acyltransferase (GPAT) (Gidda et al., 2009), and oleosins (OLE) (Chen et al., 2010b). Northern blotting technique demonstrated that Dgat2 and Fadx genes are

Abbreviations: Gapdh, glyceraldehyde 3 phosphate dehydrogenase; qPCR, quantitative real-time PCR; Rpl19b, ribosomal protein 19b; Ubl, ubiquitin protein ligase.

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predominately expressed in the developing tung seeds (Dyer et al., 2002; Shockey et al., 2006). These expression patterns were confirmed by TaqMan and SYBR Green qPCR (Cao and Shockey, 2012). However, little is known about the expression patterns of other genes in tung seeds. The study of oil biosynthesis is complicated by the facts that synthesis of these lipids requires at least 10 enzymatic steps and each step is potentially catalyzed by multiple isozymes (Cahoon et al., 2007; Dyer et al., 2008; Chapman et al., 2012). Furthermore, it has been difficult to study tung oil biosynthesis at the protein level because they are mostly hydrophobic and membrane-localized proteins (Dyer et al., 2002; Shockey et al., 2006; Cao et al., 2011, 2012).

Some of the questions raised above can be answered by quantitative real-time-PCR (qPCR); e.g., the number of isoforms of oil biosynthetic genes expressed in tung seeds, the relative expression levels of these isoforms, and how the expression patterns coordinate with tung oil biosynthesis. qPCR is widely used for gene expression analysis owing to its superior advantages compared to the traditional Northern blotting and end-point PCR (Bustin et al., 2005; VanGuilder et al., 2008). TaqMan and SYBR Green qPCR assays are two frequently used qPCR methods. TaqMan qPCR uses a fluorogenic single-stranded oligonucleotide probe that binds only the DNA sequence between the two PCR primers (Holland et al., 1991). SYBR Green qPCR uses a fluorogenic dye to intercalate into double-stranded DNA to monitor the amplification of target gene specifically initiated by gene-specific primers (Schneeberger et al., 1995). Despite the differences in the detection chemistries, both qPCR assays exhibit large dynamic range, tremendous sensitivity, high sequence-specificity, little to no post-amplification processing, and sample high throughput. However, many genetic, environmental and experimental factors affect the reliability and reproducibility of qPCR results because of the high sensitivity (Bustin, 2002). Therefore, one of the critical tasks of qPCR assay design is to select stably expressed internal reference genes for the analysis due to the inherited variations of gene expression among individual organisms, various tissues, different experimental stages and RNA stability, experimental variations such as RNA extraction methods and cDNA preparations, and human errors (Bustin et al., 2009). Carefully selected internal reference mRNAs are used to normalize transcript levels of test genes to more accurately detect the variations (Livak and Schmittgen, 2001; Pfaffl, 2001; Bustin et al., 2005; Udvardi et al., 2008).

The objective of this study was to characterize the expression of potential reference genes in the tung tree to provide a sound basis for reliable and reproducible qPCR results in later analyses. We analyzed the expression of three potential reference genes by TaqMan and SYBR Green qPCR assays using RNAs from tung seeds, leaves, and flowers following the MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments (Bustin et al., 2009). The expression of tung 60s ribosome protein L19 (Rpl19b) (Cao and Shockey, 2012), glyceraldehyde 3 phosphate dehydrogenase (Gapdh) (Han et al., 2012) and ubiquitin protein ligase (Ubl) (Pastor et al., 2013) was examined using RNA from three trees, three tissues and 11 time stages of developing seeds. We also analyzed variations of the three mRNAs in tung tissues using two RNA extraction methods and two cDNA preparations in the same or different PCR plates.

## 2. Materials and methods

### 2.1. Tung mRNA sequences, qPCR primers and TaqMan probes

Tung 60s ribosome protein L19 (Rpl19b, GenBank accession No. FJ362591) was obtained from the National Center for Biotechnology Information (NCBI)'s non-redundant protein sequence databases

(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Tung glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and ubiquitin protein ligase (Ubl) mRNA sequences were obtained from in-house sequencing projects. The qPCR primers and TaqMan probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) and synthesized by Biosearch Technologies, Inc. (Navato, CA). The  $T_{ms}$ s of the individual primer and probe are variable to a few degrees but the  $T_{ms}$ s for the probes are generally 10° higher than the corresponding primers. The names of mRNAs, the amplicon sizes and the nucleotide sequences (5'-3') of the forward primers, TaqMan probes [Dual-Labeled tetrachlorofluorescein (TET)-Black Hole Quencher-1 (BHQ1)], and reverse primers, respectively, are as follows: Gapdh: 66 bp, 5'-TGAAGCACTCCGATGTGAA-3' (forward primer), 5'-TCAAGGATGAGAAGACC-3' (probe), 5'-AG-TAACTGGCTTCTCACCAAAGAGA-3' (reverse primer); Rpl19b: 70 bp, 5'-GCGGAGAACGCGTGTCTG-3' (forward primer), 5'-CCTGCTG-CGCAAATACCGGAA-3' (probe), 5'-CATGTGCTTGTCAATTITTTGG-3' (reverse primer); Ubl: 61 bp, 5'-AGCTGGACCCAGAAGTATGCA-3' (forward primer), 5'-TGGGTTAACAGTGTGC-3' (probe), 5'-AGCCCTCCATCACACACAA-3' (reverse primer).

### 2.2. RNA isolation and cDNA synthesis

Tung tree samples were collected from the American Tung Oil Corporation orchard in Lumberton, Mississippi, frozen in liquid nitrogen and stored in -80 °C freezer. Tung tissues were ground into powder with mortar and pestle under liquid nitrogen. Total RNA were isolated from tung tree tissues with DNase treatment by Spectrum Plant Total RNA Kit (Sigma, St. Louis, MO) as described (Cao and Shockey, 2012) and the hot borate method as described (Wan and Wilkins, 1994). RNA concentrations and integrity were determined using RNA 6000 Nano Assay Kit and the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) with RNA 6000 Ladder as the standards as described (Cao and Shockey, 2012). Tung cDNA was synthesized from total RNA using SuperScript II reverse transcriptase. The cDNA synthesis mixture (20 μl) contained 5 μg total RNA, 2.4 μg oligo(dT)<sub>12-18</sub> primer, 0.1 μg random primers, 500 μM dNTPs, 10 mM DTT, 40 uRNaseOUT, and 200 u SuperScript II reverse transcriptase in 1× first-strand synthesis buffer (Life Technologies, Carlsbad, CA). The cDNA synthesis reaction proceeded at 42 °C for 50 min. The cDNA was stored in -80 °C freezer and diluted with water to 1–5 ng/μl before qPCR analyses.

### 2.3. Quantitative real-time PCR analysis

TaqMan qPCR reaction mixture contained variable amounts of total RNA-derived cDNA, the forward primer, reverse primer, TaqMan probe, and 1× Absolute QPCR Mix (ABgene House, Epsom, Surrey, UK). SYBR Green qPCR reaction mixture contained variable amounts of total RNA-derived cDNA, forward primer, reverse primer, and 1X iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). The reactions in 96-well clear plates sealed by adhesives were performed with CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad Laboratories). The thermal cycle conditions for Taq-Man assay were as follows: 2 min at 50 °C and 15 min at 95 °C (This step is required for the activation of Thermo-Start DNA polymerase, a chemically modified hot-start version of Thermoprimed *Taq* DNA polymerase, which prevents non-specific amplification), followed by 50 cycles at 95 °C for 15 s and 60 °C for 60 s. The thermal cycle conditions for SYBR Green assay were as follows: 3 min at 95 °C, followed by 40 cycles at 95 °C for 10 s, 65 °C for 30 s and 72 °C for 30 s.

The qPCR reactions for primer and probe optimization contained 5 ng RNA-equivalent cDNA from tung seeds, leaves, and flowers. For TaqMan qPCR primer optimization, the mixtures contained variable primer concentrations (10, 50, 100, 200, and 400 nM) and fixed

probe concentration (200 nM). For TaqMan qPCR probe optimization, the mixtures contained variable probe concentrations (10, 50, 100, 200, and 400 nM) and fixed primer concentration (200 nM each of the forward and reverse primers). For SYBR Green qPCR primer optimization, the reaction mixtures contained variable primer concentrations (10, 50, 100, 200, and 400 nM). The reactions for qPCR efficiency contained variable template concentrations (0.05, 0.5, 2.5, 5, 12.5, 25 ng) of RNA-equivalent cDNA from tung seeds, leaves, and flowers. The assay mixtures contained optimized concentrations (200 nM) for the primers with or without TaqMan probe.

#### 2.4. Mathematic and statistical analysis

The  $2^{-\Delta C_T}$  method of relative quantification was used to determine the fold change in expression (Livak and Schmittgen, 2001). The amplification efficiency of qPCR assay is estimated on the basis of equation  $E = (10^{-1/\text{slope}} - 1) \times 100$  (Schneider et al., 2012). The means and standard deviations were presented from 2 to 11 assays for each mRNA, each tree, each tissue, and each developmental stage analyzed by both qPCR methods.

### 3. Results

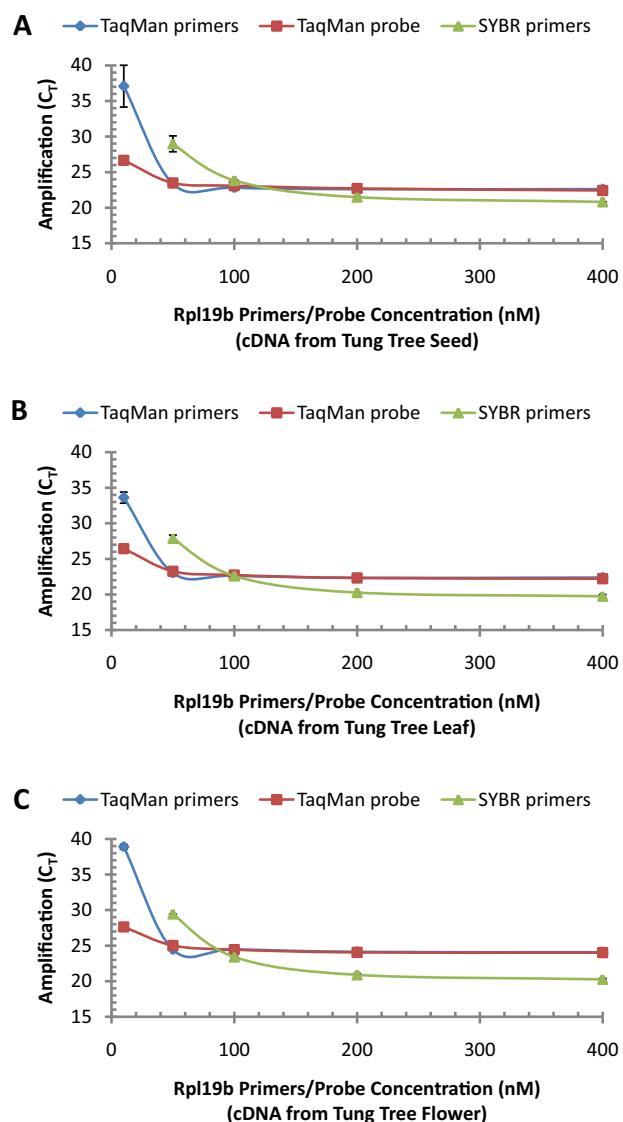
#### 3.1. qPCR optimization for reference gene expression

The concentrations of TaqMan primers, TaqMan probes, and SYBR Green primers were optimized using 5 ng RNA-equivalent cDNA from tung seeds, leaves and flowers (Fig. 1A–C). For primer optimization, qPCR reactions contained 10, 50, 100, 200, and 400 nM each of forward primer and reverse primer (TaqMan and SYBR Green qPCR assays) and fixed probe concentration at 200 nM (TaqMan qPCR assay only). For TaqMan probe optimization, the qPCR reactions contained 10, 50, 100, 200, and 400 nM of Taq-Man probe and fixed primer pair concentrations at 200 nM. Fig. 1A shows that the  $C_T$  values of TaqMan qPCR amplification were the highest when 10 nM of the primer pair or the TaqMan probe were used in the assays, but the  $C_T$  values were lower when higher concentrations of primer pair and probe were used in the assays (The higher the  $C_T$  value the less the expression level). The primer pair concentrations at 100 nM and above saturated the TaqMan qPCR reactions.

Fig. 1A also shows that SYBR Green qPCR amplification as measured by  $C_T$  value was undetectable when 10 nM primers were used and  $C_T$  value was highest when the primer pair concentrations were 50 nM, but  $C_T$  values were lower when higher concentrations of primer pair were used. Two hundred nanomolar primer pair concentrations saturated the qPCR reactions. Similar TaqMan and SYBR Green qPCR results were obtained using cDNA from tung seeds (Fig. 1A), leaves (Fig. 1B), or flowers (Fig. 1C). These results indicate that 100 nM or above of the forward primer, reverse primer, and TaqMan probe are optimal concentrations for TaqMan qPCR assay and that 200 nM or above of the forward primer and reverse primer are optimal concentrations for SYBR Green qPCR assay. The TaqMan assay was more sensitive than the SYBR Green assay since qPCR amplification products were detectable with TaqMan assays when using the lowest concentrations of the primer pair (10 nM) and saturated at 100 nM.

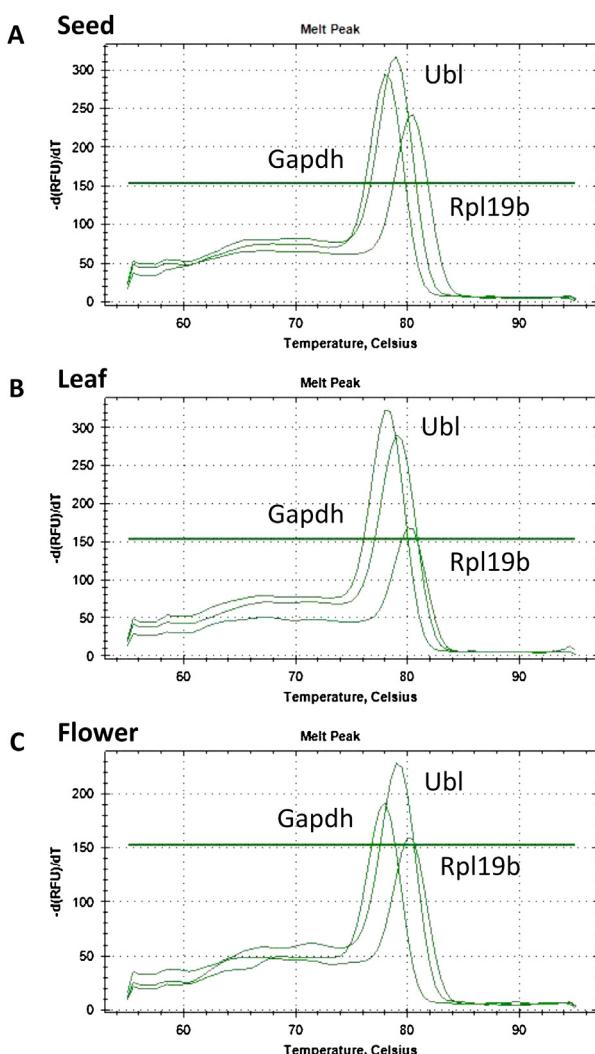
#### 3.2. Melt curve analyses for SYBR Green qPCR assays

TaqMan and SYBR Green qPCR assays use different chemistry in the assays. TaqMan uses a fluorogenic single-stranded oligonucleotide probe that binds only the DNA sequence between the two PCR primers (Holland et al., 1991). Therefore, only specific PCR product can generate fluorescent signal in TaqMan qPCR. However, SYBR Green dye intercalates into double-stranded DNA to



**Fig. 1.** TaqMan and SYBR Green qPCR optimization. The qPCR reactions contained 5 ng RNA-equivalent cDNA from tung seeds (A), leaves (B), and flowers (C), forward primer, reverse primer, TaqMan probe, and Absolute QPCR Mix (TaqMan qPCR assay) or the forward primer, reverse primer, and iQ SYBR Green Supermix (SYBR Green qPCR assay). The reactions were performed with CFX96 real-time system-C1000 Thermal Cycler. TaqMan primers refer to that the TaqMan primer concentrations were the variables and TaqMan probe concentration was fixed at 200 nM in the qPCR assay mixtures. TaqMan probe refers to that the TaqMan probe concentrations were the variables and TaqMan primer concentrations were fixed at 200 nM. SYBR primers refer to that the primer concentrations were the variables.

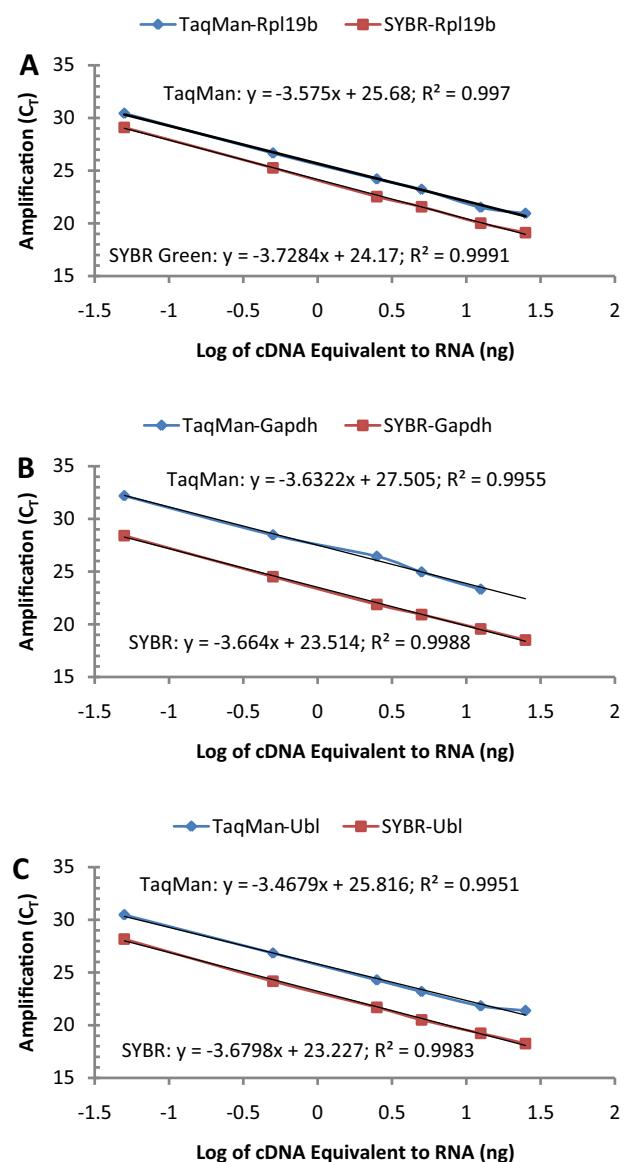
monitor the amplification of target gene specifically initiated by gene-specific primers (Schneeberger et al., 1995). Therefore, SYBR Green qPCR assays can generate false positive signals if non-specific products or primer-dimers are present in the assay. Melt curve analysis of SYBR Green qPCR assays is widely used to determine if non-specific products are formed during qPCR amplification (Han et al., 2012). Melt curve analyses shows a single melt peak in each qPCR melt curve for each target qPCR amplification using RNA from tung seeds (Fig. 2A), leaves (Fig. 2B), and flowers (Fig. 2C). Agarose gel electrophoresis shows that each reference gene-specific qPCR resulted in a single DNA fragment with predicted size of the amplicon (Cao et al., 2013). These results suggest that SYBR Green qPCR assays produce mRNA-specific amplification products for quantitative analyses of Rpl19b, Gapdh, and Ubl gene expression in tung tissues.



**Fig. 2.** Melt curve analysis of SYBR Green qPCR assays. The qPCR reactions contained 5 ng RNA-equivalent cDNA from tung seeds (A), leaves (B), or flowers (C), the forward primer, reverse primer, and iQ SYBR Green Supermix.

### 3.3. qPCR efficiency for reference gene expression

The optimized primer and probe concentrations (200 nM) were used for estimating qPCR efficiency under variable template concentrations (0.05, 0.5, 2.5, 5, 12.5, 25 ng) of RNA-equivalent cDNA from tung seeds, leaves, and flowers. Fig. 3 shows the comparison of  $C_T$  values vs. amounts of cDNA from week 6 seeds of tung tree using the primer pair and probe for Rpl19b (Fig. 3A), Gapdh (Fig. 3B), and Ubl (Fig. 3C) with TaqMan and SYBR Green qPCR assays. Both assays produced high correlation coefficients ( $r^2 > 0.99$ ). TaqMan qPCR assays generated slightly greater slopes (or less negative slope) than those of SYBR Green qPCR assays, suggesting a slightly higher sensitivity with TaqMan qPCR assay. TaqMan qPCR assays also generated higher y-intercepts than those of SYBR Green qPCR assays. The y-intercept corresponds to the theoretical limit of detection of the reaction, or the  $C_T$  value expected if the lowest copy number of target molecules denoted on the x-axis gave rise to statistically significant amplification. Similar trends of the TaqMan qPCR correlation coefficients, slopes, and y-intercepts were generated using cDNAs from various stages of developing tung seeds, leaves, and flowers, with calculated qPCR efficiencies ranging from 88% to 103% (Table 1). These results suggest that both qPCR assays are reliable methods for analyzing reference gene expression in tung tissues



**Fig. 3.** Comparison of TaqMan and SYBR Green qPCR efficiency. The qPCR reaction mixtures contained variable concentrations of RNA-equivalent cDNA from tung seeds, the optimized concentrations of each primer and probe (200 nM), and Absolute QPCR Mix (TaqMan qPCR) or iQ SYBR Green Supermix without the probe (SYBR Green qPCR). The representative results of Rpl19b (A), Gapdh (B) and Ubl (C) for the seeds are shown in the figure. The results of qPCR assays for other tissues and seed developmental stages are presented in Table 2.

and agree with previous conclusion that TaqMan qPCR is more sensitive than SYBR Green qPCR.

### 3.4. Variation of reference gene expression among tung trees

Variations among tung trees on the expression of Rpl19b, Gapdh and Ubl genes were analyzed by TaqMan and SYBR Green qPCR using cDNA from multiple stages of developing tung seeds under optimized primer pair and probe concentration at 200 nM. The  $C_T$  values and standard deviations of TaqMan assays for Rpl19b, Gapdh and Ubl were  $23.04 \pm 0.57$ ,  $23.94 \pm 1.63$ , and  $22.54 \pm 0.58$ , respectively (Table 2). The  $C_T$  values and standard deviations of SYBR Green qPCR assays for Rpl19b, Gapdh and Ubl were  $21.15 \pm 0.30$ ,  $21.83 \pm 0.53$ , and  $19.88 \pm 0.39$ , respectively (Table 2). Both qPCR assays generated similarly smaller standard deviations for Rpl19b and Ubl, but much larger standard deviations for Gapdh. These

**Table 1**

TaqMan qPCR efficiency for quantifying reference mRNAs in tung seeds, leaves and flowers.

Tung tissue	mRNA	Slope	y-Intercept	Correlation coefficient	Efficiency $E = [10(1 - S) - 1] \times 100 (\%)$
Seed (2 weeks)	Rpl19b	-3.5847	24.320	0.9990	90
	Gapdh	-3.6036	27.112	0.9958	101
	Ubl	-3.4397	24.924	0.9997	95
Seed (4 weeks)	Rpl19b	-3.2513	24.802	0.9873	103
	Gapdh	-3.3768	27.617	0.9816	98
	Ubl	-3.4679	25.816	0.9951	94
Seed (6 weeks)	Rpl19b	-3.4554	26.047	0.9992	95
	Gapdh	-3.4443	29.346	0.9985	95
	Ubl2	-3.3888	26.009	0.9976	97
Seed (10 weeks)	Rpl19b	-3.5073	25.267	0.9988	93
	Gapdh	-3.4736	30.201	0.9993	94
	Ubl	-3.6102	26.120	0.9980	89
Leaf	Rpl19b	-3.7999	26.557	0.9958	103
	Gapdh	-3.6468	29.662	0.9974	88
	Ubl	-3.4161	24.633	0.9964	96
Flower	Rpl19b	-3.3964	24.918	0.9990	97
	Gapdh	-3.4946	28.408	0.9951	93
	Ubl	-3.3607	24.197	0.9982	99

The qPCR reaction mixtures contained variable amounts of RNA-equivalent cDNAs from tung seeds (0.05, 0.5, 2.5, 5, 12.5 and 25 ng), the optimized concentrations of each primer and probe (200 nM) and QPCR Mix. The qPCR reactions were performed in 96-well plates with CFX96 real-time system-C1000 Thermal Cycler.

results suggest that Rpl19b and Ubl are very stably expressed in the three trees and better suitable as reference mRNAs in the qPCR analyses of gene expression among the tung trees. Table 2 also shows that SYBR Green qPCR generated lower  $C_T$  values than TaqMan qPCR even using less cDNA templates. Although different amounts of cDNA templates were used in the two qPCR assays, the concentrations of the templates were in the linear range of the cDNAs for the reference genes (Fig. 3). That SYBR Green qPCR generated much higher  $C_T$  values than TaqMan qPCR is in agreement with the previous observation, probable due to the non-specific binding of SYBR dye to the double-stranded DNA molecules.

### 3.5. Variation of reference gene expression among tung tissues

Variations among tung tissues on the expression of Rpl19b, Gapdh and Ubl genes were also analyzed by TaqMan and SYBR Green qPCR using cDNA from tung seeds, leaves, and flowers under optimized primer pair and probe concentration at 200 nM. The  $C_T$  values and standard deviations of TaqMan assays among the three tissues for Rpl19b, Gapdh and Ubl were  $22.71 \pm 0.23$ ,  $26.50 \pm 0.74$ , and  $22.41 \pm 0.72$ , respectively (Table 3). The  $C_T$  values and standard deviations of SYBR Green qPCR assays among the three tissues for Rpl19b, Gapdh and Ubl were  $21.34 \pm 0.30$ ,  $22.23 \pm 0.26$ , and  $19.41 \pm 1.06$ , respectively (Table 3). Both qPCR assays generated smaller standard deviations for Rpl19b, but larger standard deviations for Gapdh and Ubl. These results suggest that Rpl19b is very

stably expressed in the three tissues and better suitable as a reference mRNA than Gapdh or Ubl in the qPCR analyses of gene expression in various tung tissues.

### 3.6. Variation of reference gene expression among developmental stages of tung seeds

TaqMan and SYBR Green qPCR assays were also used to analyze the variations among various stages of developing tung seeds on the expression of Rpl19b, Gapdh and Ubl genes under optimized primer pair and probe concentration at 200 nM. The  $C_T$  values and standard deviations of TaqMan assays among the 11 stages of tung seeds for Rpl19b, Gapdh and Ubl were  $23.05 \pm 0.36$ ,  $23.90 \pm 1.21$ , and  $22.57 \pm 0.41$ , respectively (Table 4). The  $C_T$  values and standard deviations of SYBR Green qPCR assays among the 6 stages of tung seeds for Rpl19b, Gapdh and Ubl were  $21.18 \pm 0.29$ ,  $21.83 \pm 1.04$ , and  $20.08 \pm 0.17$ , respectively (Table 4). Both qPCR assays generated similar standard deviations for Rpl19b and Ubl, but much larger standard deviations for Gapdh. Similar to the results presented in Table 2, Table 4 shows that SYBR Green qPCR generated lower  $C_T$  values than TaqMan qPCR even using less cDNA templates. These results suggest that Rpl19b and Ubl are very stably expressed in different stages of developing seeds and suitable as reference mRNAs in the qPCR analyses of gene expression in developing seeds.

**Table 2**

Variation of reference gene expression among tung trees.

qPCR method	mRNA	Tree 1 ( $C_T$ )	Tree 2 ( $C_T$ )	Tree 3 ( $C_T$ )	Mean $\pm$ SD ( $C_T$ )
TaqMan	Rpl19b	22.97	22.46	23.69	$23.04 \pm 0.57$
	Gapdh	24.31	23.83	23.69	$23.94 \pm 1.63$
	Ubl	23.05	21.71	22.86	$22.54 \pm 0.58$
SYBR Green	Rpl19b	21.49	21.02	20.94	$21.15 \pm 0.30$
	Gapdh	22.40	21.71	21.36	$21.83 \pm 0.53$
	Ubl	20.18	19.44	20.02	$19.88 \pm 0.39$

The qPCR reaction mixtures contained 25 ng in 25  $\mu$ L (TaqMan qPCR) or 5 ng in 12.5  $\mu$ L (SYBR Green qPCR) of RNA-equivalent cDNAs from various stages of tung seeds, the optimized concentrations of each primer and probe (200 nM) and QPCR Mix. The  $C_T$  values under each tree represent the means of the  $C_T$  values from 11 stages of seeds (TaqMan qPCR) or 6 stages of seeds (weeks 2, 4, 5, 6, 8 and 10) (SYBR Green qPCR) with 2–4 assays for each stage.

**Table 3**

Variation of reference gene expression among tung tissues.

qPCR method	mRNA	Seed ( $C_T$ )	Leaf ( $C_T$ )	Flower ( $C_T$ )	Mean $\pm$ SD ( $C_T$ )
TaqMan	Rpl19b	22.75	22.90	22.46	22.71 $\pm$ 0.23
	Gapdh	26.19	27.35	25.97	26.50 $\pm$ 0.74
	Ubl	23.16	22.33	21.74	22.41 $\pm$ 0.72
SYBR Green	Rpl19b	21.58	21.00	21.42	21.34 $\pm$ 0.30
	Gapdh	22.50	22.21	21.98	22.23 $\pm$ 0.26
	Ubl	20.64	18.82	18.78	19.41 $\pm$ 1.06

The qPCR reaction mixtures contained 5 ng of RNA-equivalent cDNAs from various stages of tung seeds, leaves and flowers, the optimized concentrations of each primer and probe (200 nM) and QPCR Mix. The  $C_T$  values under "seed" represent the means of the  $C_T$  values from 4 stages of seeds (weeks 2, 4, 5, 6 and 10) (TaqMan qPCR) or 6 stages of seeds (weeks 2, 4, 5, 6, 8 and 10) (SYBR Green qPCR) with 2–4 assays for each stage. The  $C_T$  values under "leaf" and "flower" represent the means of the  $C_T$  values from 2 to 4 assays.

### 3.7. Variation of reference gene expression between RNA extraction methods, cDNA preparations and assayed in the same or different plates

Experimental factors and human errors also affect qPCR data analysis. We analyzed the variations of reference gene expression between RNA extraction methods, cDNA preparations and assayed in the same or different plates by qPCR assays. The  $C_T$  values and standard deviations of TaqMan assays among the 10 stages of tung seeds for the Spectrum Kit and the hot borate method were  $23.26 \pm 0.36$  and  $22.31 \pm 0.25$ , respectively. TaqMan qPCR assays generated similar standard deviations for RNA extraction by both methods. The  $C_T$  values and standard deviations of TaqMan assays using the two different cDNA preparations among the 11 stages of tung seeds for Rpl19b were  $23.42 \pm 0.63$  and  $23.57 \pm 0.59$ , respectively. TaqMan qPCR assays generated similar standard deviations for cDNAs prepared over a 4-month interval.

TaqMan and SYBR Green qPCR assays were also used to analyze the variations between wells within the same plate or different plates using RNA from several stages of developing tung seeds on the expression of Rpl19b, Gapdh and Ubl genes under optimized primer pair and probe concentration at 200 nM. The  $C_T$  values and standard deviations of TaqMan and SYBR Green qPCR assays between the wells in the same plate and different plates were very small regardless of the RNA isolated from different developing stages of tung seeds (data not shown). These results suggest that experimental error is minimal and confirm the previous conclusion that Rpl19b and Ubl are very stably expressed and suitable as reference mRNAs in the qPCR analyses of tung gene expression.

## 4. Discussion

Tung tree (*V. fordii*) is an industrial crop because it produces economically important tung oil in the seeds. Tung oil has been widely used as a drying ingredient in paints, varnishes, coatings and finishes due to eleostearic acid, the major component of tung oil (Kopacz, 1968; Pryde, 1979). New uses of tung oil have been explored as a raw material to produce biodiesel, polyurethane and wood flour composites, thermosetting polymer and repairing agent

for self-healing epoxy coatings (Park et al., 2008; Chen et al., 2010c; Samadzadeh et al., 2011; Manh et al., 2012; Aranguren et al., 2012; Liu et al., 2013). However, most of the tung orchards in the United States were destroyed by hurricanes.

Our project focuses on alternative ways of producing tung oil-like fatty acids and other high-value industrial oils by engineering tung oil biosynthetic genes into oilseed crops. Dozens of genes have been identified for tung oil biosynthesis (Dyer et al., 2002; Hwang et al., 2004; Shockley et al., 2005, 2006; Gidda et al., 2009; Chen et al., 2010b; Pastor et al., 2013). However, selection of target genes for genetic engineering of plant oils is difficult because oil is biosynthesized by at least 10 enzymatic steps and each step is probably catalyzed by multiple isozymes (Shockley et al., 2006; Cahoon et al., 2007; Dyer et al., 2008; Chen et al., 2010b, 2012a; Cao, 2011; Chapman et al., 2012).

Quantitative real-time-PCR (qPCR) would aid to the selection of target genes because it is useful to identify the number of isoforms of oil biosynthetic genes expressed in tung seeds, the relative expression levels of these isoforms, and how the expression patterns coordinate with tung oil biosynthesis. A number of factors affect the calculation of gene expression data due to the inherited variations of gene expression among individual organisms, various tissues, different experimental stages and RNA stability, experimental variations such as RNA extraction methods and cDNA preparations, and human errors (Bustin, 2002; Bustin et al., 2009). Therefore, one critical task of qPCR assay design is to select stably expressed internal reference genes for normalizing transcript levels of test genes during the post qPCR data analysis (Livak and Schmittgen, 2001; Pfaffl, 2001). The importance of selecting suitable reference genes is evident from a great number of publications in recent years (Toegel et al., 2007; Chen et al., 2010a; Ling and Salvaterra, 2011; Li et al., 2012; Pettengill et al., 2012; Shi et al., 2012; Lardizabal et al., 2012).

In this study, we optimized qPCR assays for three reference genes (Rpl19b, Gapdh, Ubl) and compared their expression levels by TaqMan and SYBR Green qPCR assays using RNAs from three trees, three tissues (seeds, leaves and flowers) and 11 time stages of developing seeds. We also analyzed variations of the three mRNAs in tung tissues using two RNA extraction methods and two cDNA

**Table 4**

Variation of reference gene expression among different stages of tung seeds.

qPCR method	mRNA	1 ( $C_T$ )	2 ( $C_T$ )	3 ( $C_T$ )	4 ( $C_T$ )	5 ( $C_T$ )	6 ( $C_T$ )	7 ( $C_T$ )	8 ( $C_T$ )	9 ( $C_T$ )	10 ( $C_T$ )	11 ( $C_T$ )	Mean $\pm$ SD
TaqMan	Rpl19b	22.69	22.92	22.57	22.66	23.16	22.81	23.18	23.28	23.69	23.14	23.51	23.05 $\pm$ 0.36
	Gapdh	23.76	22.53	22.13	22.00	23.74	24.21	24.39	24.67	24.64	25.36	25.43	23.90 $\pm$ 1.21
	Ubl	22.05	22.54	22.13	22.18	22.75	22.64	22.76	22.77	22.13	22.89	23.39	22.57 $\pm$ 0.41
SYBR Green	Rpl19b		21.10		20.69	21.11	21.53		21.39		21.27		21.18 $\pm$ 0.29
	Gapdh		21.09		20.38	21.29	22.62		22.80		22.76		21.83 $\pm$ 1.04
	Ubl		20.27		19.87	19.93	20.13		19.99		20.27		20.08 $\pm$ 0.17

The qPCR reaction mixtures contained 25 ng in 25  $\mu$ l (TaqMan qPCR) or 5 ng in 12.5  $\mu$ l (SYBR Green qPCR) of RNA-equivalent cDNAs from various stages of tung seed, the optimized concentrations of each primer and probe (200 nM) and QPCR Mix. The  $C_T$  values under each seed stage represent the means of the  $C_T$  values from three trees with 2–4 assays for each tree.

preparations, and the same or different PCR plates. We used Rpl19b and Ubl as reference genes in previous studies of gene expression in tung trees but did not compare them directly (Cao and Shockey, 2012; Pastor et al., 2013). Han et al. compared the expression of a number of potential reference genes in tung tree and identified Gapdh as the best reference gene in their studies; however, they did not examine the effectiveness of Rpl19b or Ubl in their studies (Han et al., 2012). Our overall results demonstrated that Rpl19b was the most stably expressed gene (i.e., the best reference mRNA for qPCR assays), followed closely by Ubl. This is in agreement with our general observation that genes coding for ribosomal proteins are reliable reference genes in animal cells and tissues (Cao et al., 2008, 2010).

## 5. Conclusions

One critical task of qPCR assay design is to select stably expressed internal reference genes for normalizing transcript levels of test genes during data analysis. We compared the expression of three potential reference genes (Rpl19b, Gapdh, Ubl) by Taq-Man and SYBR Green qPCR assays using RNAs from three trees, three tissues and 11 time stages of developing seeds. We also analyzed variations of the three mRNAs in tung tissues using two RNA extraction methods and two cDNA preparations, and the same or different PCR plates. Optimization studies indicated that 200 nM qPCR primers and TaqMan probes were sufficient for both TaqMan and SYBR Green qPCR assays of the reference gene expression in tung tree. Under optimized assay conditions, both qPCR assays gave high correlation coefficient and similar amplification efficiency, but TaqMan qPCR assay was more sensitive than SYBR Green qPCR. Overall results demonstrated that Rpl19b and Ubl were preferable reference genes for both TaqMan and SYBR Green qPCR assays for quantitative gene expression analysis in tung trees. The development of these tung tree reference gene assays should facilitate identifying gene targets for genetic engineering industrial oils such as tung oils in oilseed crops.

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